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14. ABSTRACT  Class IA PI3Ks are regarded the most important in regulating cell proliferation and tumorigenesis. The p55γ protein is a regulatory subunit of class IA PI3K. In vitro study has demonstrated that the NH2-terminal of p55γ is sufficient to bind the cell cycle regulatory protein pRb. Direct association between Ca++/calmodulin and p85 subunit of PI3K has been demonstrated by coimmunoprecipitation and affinity chromatography. Ca++/calmodulin directly interact with SH2 domains of p85, resulting inactivation of p110. The NH2-terminal and COOH terminal of p55γ SH2 domains are 89% and 81% identical with that of p85α, respectively. Here we addressed the issue of whether p55γ associates with calmodulin in vitro in human 293T cells. Using calmodulin-conjugated sepharose beads we analyzed the Flag-tagged p55γ cDNA transfected cells. The cell cycle profile of HEK293 cells stable expressing Flag-tagged p55γ were analyzed by flow cytometry. Our experiments demonstrated that overexpression of p55γ in HEK293 cells promoted cell cycle progression. Our data also show a calcium-dependent calmodulin-p55γ interaction in human 293T cells, and the overexpression of FLAG-tagged p55γ stabilized the interaction between calmodulin and retinoblastoma protein. We propose that p55γ protein regulate cell cycle progression through formation of a ternary complex with calmodulin and Rb.					
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**Introduction:**

Polypeptide growth factors and steroid hormones regulate cell proliferation, cell growth, and cell survival, and are important in both the etiology of breast cancer and as targets of its therapy. Central to the actions of both growth factors and steroids are control of cellular phospholipid metabolism,  $\text{Ca}^{++}$  metabolism, and protein kinase/phosphatase cascades. Interaction of  $\text{Ca}^{++}$  metabolism with phospholipids and protein kinases is a complex, understudied area, but also one with promise for new avenues of cancer therapy, since  $\text{Ca}^{++}$  is required for cell cycle progression.

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that generate second messengers upon receptor tyrosine kinase activation, through phosphorylation of phosphatidylinositols at the 3' position of the inositol ring. These second messengers govern cell morphology in addition to cellular activities including proliferating, survival and motility (Bader et al, 2005). The PI3K family contains three classes with multiple subunits and isoforms. Among the three classes, class IA PI3K proteins are regarded the most important in regulating cell proliferation and tumorigenesis. Class IA catalytic subunits, with p110 $\alpha$  being the most widely studied, are constitutively associated with an adaptor subunit to form a heterodimeric complex (Cantley et al, 2002).

Each of the regulatory subunits of PI3K has three major functions. Each binds and stabilizes the catalytic subunit, induces lipid kinase activity upon insulin stimulation, and confers inhibitory effects on the p110 kinase activity in the basal state (Chen et al, 2004). The p55 $\gamma$  protein is a regulatory subunit of class IA PI3K, encoded by *pik3r3* gene. When expressed in Chinese Hamster Ovary (CHO) cells, p55 $\gamma$  showed both basal and insulin-stimulated tyrosine phosphorylation (Pons et al, 1995). Human p55 $\gamma$  binds IGF1R, IR-1 and IRS-1 upon receptor activation in NIH3T3 cells (Dey et al, 1998). Its N-terminal 30 amino acids are unique among PI3K regulatory subunits. It contains an YXXM sequence, which is a putative SH2-binding motif; this motif may contribute to the specificity of its function (Dey et al, 1998). *In vitro* study showed that NH<sub>2</sub>-terminal 24 amino acid of p55 $\gamma$  is sufficient to bind the cell cycle regulatory protein pRb (Xia et al, 2003). The speculated function of the overexpressed 24 amino acid residue was to serve as a dominant-negative module, blocking the endogenous p55 $\gamma$ -Rb binding in MCF-7 cells, and leading to cell cycle arrest (Xia et al, 2003). It is worth mentioning that Rb also binds HA-tagged p55 $\alpha$  expressed in monkey kidney COS-7 cells and mouse NIH3T3 cells (Xia et al, 2003), however, the association between Rb and p85 $\alpha$ , or Rb and p50 $\alpha$  was not detected in the same system. So far there is no evidence on the direct interaction between p55 $\alpha$  and CaM, and we are more interested in p55 $\gamma$  because among the regulatory subunits, p55 $\gamma$  is encoded by a distinct gene, whereas p55 $\alpha$  is encoded by the same gene as p85 $\alpha$  and p50 $\alpha$ .

The retinoblastoma (*rb*) gene was the first tumor suppressor gene identified by its involvement in hereditary retinoblastoma. Accumulating evidence from *in vitro* and *in vivo* studies have confirmed that its gene product, a 105 KDa protein, is implicated in cellular functions including cell proliferation, differentiation, and apoptosis (Classon et al, 2002). The major function of Rb is as a cell cycle inhibitor; cells undergo arrest in the G1 phase of the cell cycle upon the activation of Rb. Rb directly binds and inhibits the transcriptional activity of E2F family members by recruitment of several chromatin-remodeling complexes to promoter regions, and results in chromatin condensation and transcription inhibition (Harbour et al, 2000). The function of Rb depends on its

phosphorylation status. The hypophosphorylated Rb is the active form, which has growth suppressive activity, and phosphorylated Rb is inactive and unable to bind E2F transcription factors (Goodrich et al, 1991).

p55 $\gamma$  selectively binds hypophosphorylated Rb in MCF-7 cells. Stronger binding of p55 $\gamma$  was selectively detected when MCF-7 cells were treated with Heregulin, a differentiation factor, compared to serum-starved, quiescent cells (Xia et al, 2003). Addition of mitogens disrupts the interaction, and the time course of complete dissociation of the p55 $\gamma$ -Rb complex is consistent with the time course of Rb phosphorylation, suggesting that there is a mechanistic connection between the two events (Xia et al, 2003).

Calmodulin senses the cellular calcium concentration, binds to and then relays the growth factor-dependent signals from epidermal growth factor receptor (Li et al, 2004a) and the Her2/Neu/ErbB2 receptors (Li et al, 2004b). Although calcium-independent calmodulin signaling also exists in the cells, here we will at least emphasize initially the calcium-dependent activation pathway, because calcium/calmodulin plays an important role in regulating cell cycle transitions. In late G1, it functions *via* cyclin D1/cdk4 to hyperphosphorylate Rb, thus releasing the E2F transcription factor (Kahl and Means, 2003). Direct association between Ca<sup>++</sup>/calmodulin and the p85 regulatory subunit of PI3K has been demonstrated by co-immunoprecipitation and affinity chromatography (Jayal et al, 1997). Ca<sup>++</sup>/calmodulin directly interact with SH<sub>2</sub> domains of p85, resulting in activation of p110. The NH<sub>2</sub>-terminal and COOH terminal of p55 $\gamma$  SH<sub>2</sub> domains are 89% and 81% identical with that of p85 $\alpha$ , respectively. However, direct interaction between p55 $\gamma$  and Ca<sup>++</sup>/calmodulin has been understudied and was not proven so far.

### **Body:**

#### **Overall Hypothesis:**

We hypothesize that calmodulin forms a ternary complex with p55 $\gamma$  and retinoblastoma protein (Rb), such that activated calmodulin modulates Rb phosphorylation and cell cycle progression through p55 $\gamma$ .

#### **Working Hypothesis 1: P55 $\gamma$ directly associates with both CaM and Rb, forming a ternary complex.**

**Task 1.** Determine if Sf9-generated p55pik binds CaM in the presence of calcium.

- a. Generate viral constructs for GST-p55pik protein expression.
- b. Transfect the insect Sf9 cells with plasmid-containing baculovirus, analysis of the transfected cells with anti-GST and anti-Np55pik antibodies for protein expression.
- c. Purify the verified GST-p55pik recombinant protein with glutathione-sepharose beads.
- d. Pass the purified protein through a CaM sepharose column in the presence of Ca<sup>++</sup>, elute the column and assay the fractions by western blotting.

In order to test the first working hypothesis, I first performed the experiments to determine that p55 $\gamma$  overexpressed in human embryonic kidney (HEK) 293 cells indeed binds to calmodulin. The major features of the HEK 293 cell system as compared to

previously proposed insect cell system are (i) the human cell expression system which likely express p55 $\gamma$  as its native endogenous form and (ii) well-documented high transfection efficiency and protein expression. The association between p55 $\gamma$  and calmodulin has been further confirmed in the human breast cancer AU565 cells, using a calmodulin pull-down assay (Fig. 1).

The biological mechanisms underlying how p55 $\gamma$  binds to CaM are important. If we identify specific binding domains, novel therapeutics which inhibit tumor cell progression by blocking interaction of p55 $\gamma$  and CaM could be developed in treating human breast cancer. Using a computer search, putative CaM binding sites have been identified in the SH2 domains of p55 $\gamma$  sequence, which are conserved among p55 $\gamma$ , p85 $\alpha$ , and p85 $\beta$  (Fig. 2). Furthermore, literature indicated a direct, calcium-dependent binding between SH2 domains of p85 and CaM (Joyal et al, 1997), which enabled us to propose a direct binding between SH2 domains of p55 $\gamma$  and CaM. Interestingly, the laboratory of Dr. David Sacks used a GST pull-down assay, and confirmed that the major CaM binding site on p85 was located in the C-SH2 domain, while the N-SH2 domain binds with less strength (Joyal et al, 1997). This result was contradictory to the prediction of CaM Target Database (Fig.2), which predicted the strongest binding site to be located at the N-SH2 domain, and the weaker site to be located at the C-SH2 domain. A third binding was predicted in between the p110 binding site and the C-SH2 domain. We are using two different approaches: (i) we have generated synthetic peptides from the predicted N-SH2 domain-binding site to block the CaM-p55 $\gamma$  interaction in the CaM pull-down assays (ongoing experiments); (ii) we are also generating the plasmids expressing p55 $\gamma$  deletion mutants with the coding sequences of the three predicted binding sites deleted separately. These mutated constructs will be used in determination of the possible calmodulin binding sites.

## Methods

**1. Construction of plasmids**—full-length cDNA encoding human p55 $\gamma$  was purchased from NIH Mammalian Gene Collection (MGC) (Invitrogen). All full-length and truncated constructs were generated *via* PCR amplification. The forward primer used for generation of N-terminal tagged p55 $\gamma$  contains the sequence coding for an additional DYKDDDK prior to the start codon of *pik3r3*. The truncated constructs are generated by two-step PCR with the truncation sites indicated as in Figure. 2. The PCR products were cloned into pcDNA3.1/V5-His TOPO TA (Invitrogen). For the constructs expressing a V5-epitope and His-tag, the stop codon at 3' of *pik3r3* is mutated. All constructs are verified by sequencing.

**2. Cell culture and transfection**--- Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's Medium (GIBCO) with 10% heat-inactivated fetal bovine serum. Human breast cancer AU565 cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. Human breast cancer MCF-7 cells were maintained in IMEM medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. For 293T and MCF7 cells, DNA transfections were carried out using FuGENE 6 (Roche Applied Science), according to manufacture's specifications. For AU565 cells, nucleofection (Amaxa) was performed with 1.5  $\mu$ g of siRNA (Dharmacon).

**3. Calmodulin pull-down assay and Immunoblotting---**293T cells were transfected with pcDNA3.1-Flag55, or non-tagged p55, or a control vector that expresses an N-terminal Flag tagged protein (pFlagSOX9). Cells were serum-starved for 24 hrs before lysis with proteinase-inhibitor cocktail-supplemented lysis buffer. Fifty  $\mu$ l of CaM-conjugated sepharose beads (BIOMOL) were incubated with 500 $\mu$ l of cell lysate in the presence of or absence of calcium for 2hrs at 4°C, glutathione sepharose beads were used as non-CaM-conjugated control for beads. Beads were boiled in reducing buffer after extensive wash with calcium-containing or calcium-free buffer to elute the bound proteins. The proteins were resolved with 12% NuPAGE gel (Invitrogen) and transferred to a PVDF membrane. The membranes were blotted with anti-flag (gift from Dr. Chen-yong Lin), anti-Rb (Cell Signaling), or anti-actin antibody (Sigma) (Fig.3).

**4. Immunoprecipitation and Immunoblotting---**293T and/or MCF7 cells will be transfected with p55 $\gamma$  constructs expressing V5 epitope tag, full-length or mutated (Fig.2). V5 is an epitope tag composed of a 14 residue peptide, GKPIPNPLLGLDST, derived from the P/V proteins of paramyxovirus SV5. Transfection of mutated p55 $\gamma$  is used to identify the interacting site of CaM on p55 $\gamma$  by disrupting the CaM-p55 $\gamma$  interaction. Cell lysates will be prepared as described, and lysates will be precleared with protein A-agarose before incubation with anti-V5 antibody or control mouse IgG overnight at 4°C. Then bound protein will be immunoprecipitated with 50 $\mu$ l of protein A-agarose for 2hr at 4°C. The protein will be resolved, transferred and blotted with anti-CaM, anti-Rb, and anti-p55 $\gamma$  antibody.

**Preliminary results---** CaM pull-down assay showed that Flag-tagged p55 $\gamma$  overexpressed in 293T cells interacts with calmodulin, when calcium is added to the reaction, more p55 $\gamma$  was pulled down by CaM beads, suggesting these proteins interact in a calcium-dependent manner. Compared to the cells expressing p55 $\gamma$ , CaM pulls down more Rb protein than control Flag-tagged protein-expressing cell lysates, suggesting the formation of the CaM-p55 $\gamma$ -Rb protein complex. CaM beads do not pull down Flag-tagged control protein, suggesting that the interaction is specific. The control sepharose beads do not bind the Flag-p55 $\gamma$ , or the Rb.

**Expected results for anti-V5 immunoprecipitation and backup strategies**

I expect that, in one of the three deletion mutant p55 $\gamma$ -expressing vectors transfected cell lysates, immunoprecipitation using anti-V5 epitope antibody, will pull down less calmodulin than the full-length p55 $\gamma$ , and that this mutant contains a deletion of the correct CaM binding site. If the binding site can not be identified in this way, I will generate and purify GST fusion protein for each of the functional domains of p55 $\gamma$ . Each individual GST fusion protein will be incubated with MCF7 cell lysates and let flow through glutathione sepharose column to identify the binding proteins.

**Task 2.** Investigate using microscopic live image, if p55pik binds CaM in the erbB2/HER2 receptor-expressing AU565 and SKBr3 breast cancer cells. (Ongoing work)

- a. Generate eCFP, or eYFP expressing constructs containing cDNA of p55pik, and CaM, respectively.
- b. Transiently transfect AU565 and SKBr3 cells with the constructs and analysis of the protein expression as well as the protein biological activities.

- c. Generate AU565 or SKBr3 cell lines stable expressing both CaM-eYFP, and p55pik-eCFP recombinant proteins
- d. Assay the protein-protein interaction using FRET with fluorometry, video-microscopy and confocal microscopy.

**Hypothesis 2: Activated calmodulin, by binding p55pik, phosphorylates Rb and causes cell cycle progression.**

**Task 3.** Determine if overexpression of p55pik results in cell cycle progression in breast cancer cells with activated CaM.

- a. Transfect MCF-7 cells with plasmid pCDNA3.1, containing the cDNA of human p55pik. Select stable expressing cell lines using the G418 selection method. (months 1-3) ---Completed
- b. Screen positive clones with anti-Np55pik antibodies. (months 4-5)---Completed
- c. Pool the positive clones and synchronize the cells, stimulate cells with growth factors and analyze cell cycle progression profile with flow cytometry (months 6-12)---Ongoing
- d. Detect the activation status of Rb in these cell lysates using anti-phospho Rb antibodies (Cell Signaling, Beverly, MA). (months 13-18)---Ongoing

Preliminary experiments using HEK293 cells stable expressing N-terminal Flag-tagged p55 $\gamma$  showed a cell cycle profile distinct from that of HEK293 cells expressing vehicle vector (Fig.5). The similar cell cycle promoting effect was also confirmed in His/V5 tagged p55 $\gamma$  transfected A1N4 cells (Fig.6).

**Task 4.** Determine if knockdown of p55pik protein expression by RNA interference leads to cell cycle arrest in breast cancer cells with activated CaM.

- a. Pilot study for the optimal transfection efficiency of four individual SiRNAs against p55pik using real-time PCR and anti-Np55pik antibodies. (months 1-6)---Completed
- b. Transfect the selected SiRNAs into AU565 cells and analyze the interference of p55pik protein expression, Rb phosphorylation status, and the apoptosis status of the cells. (months 7-24)---Ongoing

**Knockdown protein expression using small interfering RNA** ---Four pairs of Small interfering RNAs directed against *pik3r3* were designed and synthesized at Dharmacon (Chicago, IL). AU565 breast cancer cells expressing endogenous p55 $\gamma$  were transfected with 1.5  $\mu$ g of siRNA against *pik3r3*. Non-targeting siRNA was transfected as control. Seventy-two hrs post-transfection, cells were lysed and the lysates were resolved, transferred and blotted with anti- p55 $\gamma$  or anti-actin. For future studies, the apoptosis status of these cells will be detected using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI), and PARP cleavage assay (Calbiochem).

**Preliminary siRNA data** in AU565 cells showed that p55 $\gamma$  protein expression is successfully knocked down with the proper siRNA combination (Fig.4). In a previous study of mammary involution using an immortalized mammary epithelial cell line (KIM-2), when p55 $\alpha$  and p50 $\alpha$  was overexpressed, Akt phosphorylation was down-regulated (Abell et al, 2005). The inhibitory function of these PI3K regulatory subunits seems to contribute to this effect. Therefore, an inhibitory effect of p55 $\gamma$  on Akt activation is expected. No data are currently published concerning the Rb mutation status in AU565



cells, so we presume that functional Rb exists in AU565 cells. During serum starvation, p53γ binds to hypophosphorylated Rb in quiescent cells; we hypothesize that upon growth factor stimulation, p53γ facilitates the hyperphosphorylation of Rb by Ca<sup>++</sup>/CaM activation, most probably through Cyclin D/cdk4. When p53γ is down regulated, Rb phosphorylation is down regulated. In a preliminary experiment, we did not observe a significant decrease in phosphoRb signal compare to total Rb at 20 minutes time point after serum stimulation (Data not shown), possibly because we missed the time point for detection of Rb phosphorylation. We now conduct careful time course experiments post stimulation.

**Cell cycle analysis by flow cytometry**---MCF7 cells transfected with p53γ siRNA will be synchronized and subjected to flow cytometry for cell cycle profile analysis. Cells are grown on 60mm dishes with growth medium and synchronized by double thymidine block, cells are released by addition of fresh complete medium and harvested with trypsin-EDTA. After one to ten hours of stimulation, cells are washed in PBS and pelleted. After removing the wash buffer, the pellets are vortexed and resuspended in 0.1 ml of citrate/DMSO buffer ( 250 mM sucrose, 40 mM Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> 2H<sub>2</sub>O, 5% DMSO, pH 7.60). The pellets are then sent to cell cycle analysis by flow cytometry.

**Expected Results and Backup Strategies**

Transfection of siRNA against p53γ in AU565 cells will be repeated. Cells will be harvested at different time points after growth factor stimulation. I expect that at some time point after stimulation, a decreased phosphorylation of Rb be observed in p53γ knockdown cells. Also in these cells at this time point, we should detect less percentage of cells in S phase of cell cycle. Apoptosis assay will show that in p53γ knockdown cells, the anti-apoptosis effect of Rb is impaired because of the down-regulation of phosphorylation. Backup strategy for this part of the experiments will be to pre-incubate the AU565 cells with W7, a CaM inhibitor, to see if the CaM activation is the only determinant on Rb phosphorylation in this cell line.

**Key research accomplishments:**

- A calcium dependent association between p53γ and CaM was established in both human 293T and mammary carcinoma AU565 cells by CaM pull-down assay.
- Successfully knockdown p53γ at protein level by small interfering RNA method.
- Data suggested a cell cycle promoting effect of p53γ in HEK293 cells and A1N4 cells.

**Reportable Outcomes:**

- MCF7 cells stable expressing a N-terminal Flagged p53γ, or a C-terminal His and V5 tagged p53γ were established
- Poster presentation at DOD BCRP Annual Meeting, Philadelphia, PA June 8-11, 2005
- Poster presentation at 8<sup>th</sup> Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center. February 15-17, 2006

- Presented some data supported by this grant on first Ph.D. graduate student committee meeting in Dec 2005
- Thesis proposal based on the research supported by this grant was deposited and approved by Georgetown University Graduate School on May 30, 2006

### **Conclusions:**

Discovery of a functional p55 $\gamma$ -CaM interaction that leads to Rb phosphorylation and cell cycle progression will identify p55 $\gamma$  as a protein that plays important role in cell proliferation. This will possibly establish a foundation for the development of a new anti cancer drug that works synergistically with other drugs against human breast cancer.

### **References:**

1. **Bader, A. G., S. Kang, L. Zhao, and P. K. Vogt.** 2005. Oncogenic PI3K deregulates transcription and translation. *Nat.Rev Cancer* **5**:921-929.
2. **Cantley, L. C.** 2002. The phosphoinositide 3-kinase pathway. *Science* **296**:1655-1657.
3. **Chen, D., F. Mauvais-Jarvis, M. Bluher, S. J. Fisher, A. Jozsi, L. J. Goodyear, K. Ueki, and C. R. Kahn.** 2004. p50alpha/p55alpha phosphoinositide 3-kinase knockout mice exhibit enhanced insulin sensitivity. *Mol.Cell Biol* **24**:320-329.
4. **Classon, M. and E. Harlow.** 2002. The retinoblastoma tumour suppressor in development and cancer. *Nat.Rev Cancer* **2**:910-917.
5. **Dey, B. R., R. W. Furlanetto, and S. P. Nissley.** 1998. Cloning of human p55 gamma, a regulatory subunit of phosphatidylinositol 3-kinase, by a yeast two-hybrid library screen with the insulin-like growth factor- I receptor. *Gene* **209**:175-183.
6. **Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. Lee, and W. H. Lee.** 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**:293-302.
7. **Harbour, J. W. and D. C. Dean.** 2000. Rb function in cell-cycle regulation and apoptosis. *Nat.Cell Biol* **2**:E65-E67.
8. **Joyal, J. L., D. J. Burks, S. Pons, W. F. Matter, C. J. Vlahos, M. F. White, and D. B. Sacks.** 1997. Calmodulin activates phosphatidylinositol 3-kinase. *J Biol Chem* **272**:28183-28186.
9. **Kahl, C. R. and A. R. Means.** 2003. Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocr.Rev* **24**:719-736.
10. **Li, H., M. J. Ruano, and A. Villalobo.** 2004. Endogenous calmodulin interacts with the epidermal growth factor receptor in living cells. *FEBS Lett.* **559**:175-180.
11. **Li, H., J. Sanchez-Torres, A. Del Carpio, V. Salas, and A. Villalobo.** 2004. The ErbB2/Neu/HER2 receptor is a new calmodulin-binding protein. *Biochem.J* **381**:257-266.
12. **Xia, X., A. Cheng, D. Akinmade, and A. W. Hamburger.** 2003. The N-terminal 24 amino acids of the p55 gamma regulatory subunit of phosphoinositide 3-kinase binds Rb and induces cell cycle arrest. *Mol.Cell Biol* **23**:1717-1725.

## **Appendices:**

### **Abstract presented during this report period**

1): Towards identification of human p55pik interacting proteins: production and characterization of p55pik recombinant proteins and antibodies.

Youhong Wang, Tushar Deb and Robert Dickson DOD BCRP meeting June8-11, 2005

Insulin-like growth factor (IGF)-1 is one of the most potent mitogens to breast cancer cells. A majority of breast cancers express the IGF-1 receptor. Among the proteins along the IGF-axis, p55pik plays a special role as a regulatory subunit of phosphatidylinositol-3 kinase (PI3K). Although its pathophysiologic function is unknown, the mRNA level of p55pik is higher in estrogen receptor positive than negative breast cancer cells. In order to explore biological functions of p55pik, it is necessary to identify its interacting proteins. Towards this goal, we are beginning to establish an immunoprecipitation system by generating recombinant p55pik protein and p55pik-specific antibodies.

We constructed three different eukaryotic expression plasmids containing P55pik, either with or without a tag (to produce Flag or EGFP protein) for transfections, and expressed p55pik in three mammalian cell lines (MCF-7, 293T and COS-7). Two prokaryotic expression constructs were designed and used to produce p55pik-GST fusion and MBP fusion proteins. SDS-PAGE, Western blots, and flow cytometry were performed to characterize their expression. We also designed an 18 amino acid peptide antigen (DDADWREVMMPYSTEILF) derived from the N-terminal region of p55pik, which is unique to p55pik in the PI3K family. Rabbit antisera were collected monthly during the four-month period with three times of immunizations. ELISA was used to determine the titers (1:10,000) of antibodies specific for p55pik after the last booster. The specific antibodies (IgG) were isolated by affinity chromatography using a protein-A column.

We successfully expressed the IPTG-inducible GST-p55pik fusion protein in BL21(DE3) bacteria. The SDS-PAGE of IPTG induced bacteria lysates revealed a protein band of about 82 KDa, which is consistent with the theoretical molecular mass of GST protein (27Kda) combined with p55pik (55KDa). We also expressed EGFP-p55pik fusion proteins in MCF-7 and obtained a cell line stably expressing P55pik. Immunoblot analysis of cell lysates of MCF-7 and COS-7 showed a specific band at 82 KDa recognized by the anti-GFP monoclonal antibody. The molecular mass is consistent with the theoretical molecular mass of GFP (27KDa) combined with p55pik. The expression was also confirmed by flow cytometry, using an anti-GFP antibody. In addition, the p55pik-specific rabbit antiserum was purified by affinity chromatography. We found that the p55pik-specific polyclonal antibody has no cross-reaction with p85 proteins in the same PI3K family. Interestingly, our preliminary experiments suggested that calmodulin (CaM) might be a protein interacting with p55pik in both mouse and human breast cancer cells.

In the present work, we have successfully constructed and expressed recombinant p55pik proteins and established cell lines overexpressing p55pik. We also produced

rabbit antibodies specific for the N-terminal of p55pik. This work has established a foundation for further identification of the p55pik-associated proteins, which would help us understand the biological functions of p55pik. More importantly, it will provide new molecular targets for breast cancer therapy.

2) Youhong Wang, Tushar Deb, Robert Dickson. Interaction of p55gamma, a regulatory subunit of class IA PI3K, with calmodulin in 293T cells. 8<sup>th</sup> Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center. February 15-17, 2006

The p55 $\gamma$  protein is a regulatory subunit of class IA PI3K. Class IA PI3Ks are regarded the most important in regulating cell proliferation and tumorigenesis. This class of lipid kinases encompasses multiple catalytic and regulatory subunits. *In vitro* study has demonstrated that the NH<sub>2</sub>-terminal of p55 $\gamma$  is sufficient to bind the cell cycle regulatory protein pRb. Calmodulin senses the cellular calcium concentration, binds to and relays the growth factor-dependent signals from epithelial growth factor receptors and hormone receptors. Direct association between Ca<sup>++</sup>/calmodulin and p85 regulatory subunit of PI3K has been demonstrated by coimmunoprecipitation and affinity chromatography. Ca<sup>++</sup>/calmodulin directly interact with SH2 domains of p85, resulting in activation of p110. The NH<sub>2</sub>-terminal and COOH terminal of p55 $\gamma$  SH2 domains are 89% and 81% identical with that of p85 $\alpha$ , respectively. The possible association between p55 $\gamma$  and calmodulin, its impact on cell cycle progression and the related biological functions has not been studied. Here we addressed the issue of whether p55 $\gamma$  associates with calmodulin *in vitro* in human 293T cells. Using calmodulin conjugated sepharose beads, we analyzed the Flag-tagged p55 $\gamma$  cDNA transfected cells. The pull-down experiments were performed in the presence or absence of calcium in order to identify the nature of the interaction. The 293 cells stable expressing Flag-tagged p55 $\gamma$  were analyzed by flow cytometry for cell cycle profile. Our experiments demonstrated that overexpression of p55 $\gamma$  in 293 cells promoted cell cycle progression. Our data also show a calcium-dependent calmodulin-p55 $\gamma$  interaction in human 293T cells, and the overexpression of FLAG-tagged p55 $\gamma$  stabilized the interaction between calmodulin and retinoblastoma protein. We propose that p55 $\gamma$  protein regulate cell cycle progression through formation of a ternary complex with calmodulin and Rb. Detailed biological implications of this interaction require further investigation.

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### Curriculum Vitae

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## **Education**

### **Ph.D. Student** 01/2003-present

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Thesis: Characterization of p55 $\gamma$ , a novel calmodulin binding protein, and its role in cell cycle progression of human breast cancers.

Advisor: Dr. Robert B. Dickson

### **Ph.D. Student** 08/2000 – 12/2002

Microbiology and Immunology, Department of Pathobiology, University of Illinois at Urbana-Champaign, IL

Thesis: Cloning, Expression, Localization and Functional Study of Na<sup>+</sup>/H<sup>+</sup> Antiporter in *Leishmania major*

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## **Professional Employment**

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**Graduate Research Assistant** (2000-2002) Department of Pathobiology, University of Illinois at Urbana-Champaign, Illinois

**Medical Consultant** (1998-2000) Editors Office, Journal of Family Medicine, Guangzhou, China

**Visiting Instructor** (1998-2000) SUMS Extension, Sun Yat-sen University of Medical Sciences

**Assistant Engineer (part-time)** (1993) Department of Nutrition, Nestle Dairy Company China

## **Current Projects**

**Characterization of p55 $\gamma$ , a novel calmodulin binding protein, and its role in cell cycle progression of human breast cancers.**

Major technology: PCR and DNA cloning, prokaryotic and eukaryotic gene expression in *E. coli*. and mammalian cells *in vitro*, SDS-PAGE, Western-blot, cell culture, immunoprecipitation, immunoflorescent microscopy, flow cytometry, luciferase assay, cell proliferation assay.

### **Honors and Awards**

2000 Outstanding University Graduate, Sun Yat-sen University of Medical sciences  
1999 First Pharmacy of Japan Awards, Sun Yat-sen University of Medical Sciences  
1998 The United Pharmacy Awards, Sun Yat-sen University of Medical Sciences  
1998 Annual Scholarship awards, Sun Yat-sen University of Medical Sciences  
1995,1996 Annual Scholarship awards, Harbin Medical University

### **Teaching Experience**

Passed SPEAK exam (2001), a qualification exam for graduate teaching assistant in the University of Illinois at Urbana-Champaign  
Visiting English Instructor (1998-2000), SUMS Extension, Sun Yat-sen University of Medical Sciences

### **Publications**

#### **Articles in Journals**

1. Szajnman SH, Montalvetti A, **Wang Y**, Docampo R, Rodriguez JB. Bisphosphonates derived from fatty acids are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. *Bioorg Med Chem Lett*. 2003 Oct 6; 13(19):3231-5.
2. R. Martinez, **Y. Wang**, R. Docampo, etc. A Proton Pumping Pyrophosphatase in the Golgi apparatus and Plasma Membrane Vesicles of *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology*. 2002 Apr 9; 120(2); 205-213
3. **Y. Wang** Cloning, Expression and Antigenic Analysis of Sexual Stage Antigen *Pfs25* and *Pfs48/45* from *Plasmodium falciparum* Isolate *PFD-3/YN*. Master Degree Thesis. 2000. Sun Yat-sen University of Medical Sciences Press
4. **Y. Wang**, X Yu, H Chen Sequencing and Homologous Analysis of the Sexual Stage *Pfs25* gene from *Plasmodium falciparum* *PFD-3/YN* Isolate. *Chinese Journal of Zoonoses (Zhongguo Ren Shou Gong Huan Bing Zazhi)* 2000;16(6): 19 – 20.
5. H Chen, **Y. Wang** Expression in Hela Cell Line and Identification of Two Genes Coding for *EBA-175* and *HRP-II* from *FCC-1/HN* Strain of *Plasmodium falciparum*. *Chinese Journal of Zoonoses (Zhongguo Ren Shou Gong Huan Bing Zazhi)* 1999;16(5): 38-40.
6. J Yin, **Y. Wang** etc. (1999) Gene Mapping of Benign Familial Infantile convulsions in a Chinese BFIC Family. *Journal of Applied Clinical Pediatrics (Shiyong Erke Linchuang Zazhi)* 14(4): 219-222.
7. **Y. Wang** (1998) The Development and Prospect of Malaria Transmission Blocking Vaccine. *Foreign Medical Sciences (Guowai Yixue)*, Section of Parasitic Diseases. 25(6): 245-249.
8. S Luo, J Fang, **Y. Wang** etc. (1998) Production and Identification of Monoclonal Antibody for the Antigen of *Plasmodium falciparum*. *Annual bulletin of the society of parasitology (Ji Sheng Chong Xue Nian Bao)* 20:18-20.

***Abstracts and Presentations***

Y. Wang, S. Luo, Xinbing Yu. Cloning and Expression of the Gene Coding for the Sexual Stage-Specific Antigen of *Plasmodium falciparum*, Southern China Isolate FCC-1/HN. IX International Congress of Parasitology (ICOPA IX) Makuhari Messe, Chiba, Japan August 24-28, 1998

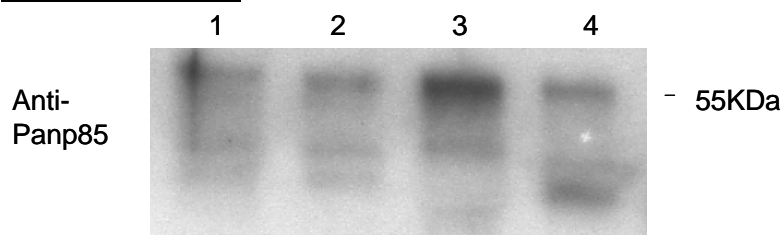
Y. Wang, R. Docampo. Cloning, expression and functional characterization of a Na<sup>+</sup>/H<sup>+</sup> exchanger in *Leishmania major*. The fifth annual CMB /MB Research Symposium, Urbana, Illinois, U.S.A. October 25, 2002

Youhong Wang, Tushar Deb, Robert Dickson. Identification of Human P55pik Interacting Proteins: Production and Characterization of P55pik Recombinant Proteins and Antibodies. 7<sup>th</sup> Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center. February 9-11, 2005

Youhong Wang, Tushar Deb, Robert Dickson. Towards identification of human p55pik interacting proteins: production and characterization of p55pik recombinant proteins and antibodies. Department of Defense Breast Cancer Research Program (DOD, BCRP) the 4<sup>th</sup> Era of Hope Annual Meeting, Philadelphia, Pennsylvania. June 8-11, 2005

Youhong Wang, Tushar Deb, Robert Dickson. Interaction of p55gamma, a regulatory subunit of class IA PI3K, with calmodulin in 293T cells. 8<sup>th</sup> Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center. February 15-17, 2006

**Supporting Data:**



**Figure 1. Calmodulin specifically binds to p55  $\gamma$  in a calcium-dependent manner.** AU565 cells were serum-starved, cell lysates were incubated with 50  $\mu$ l of calmodulin-sepharose beads in the absence (Lane 2), or in the presence (lane 3) of 0.1mM CaCl<sub>2</sub> and 1mM EGTA plus 0.1mM CaCl<sub>2</sub> (lane 4) for 2 hrs. Lysates incubated with non-conjugated sepharose were used as a negative control (lane 1). Washed beads were resolved by SDS-PAGE and transferred to PVDF membrane. Blots were probed with anti-p85pan pAb from Santa Cruz Biotechnology (CA), which recognizes p85alpha, p85beta, and p55 $\gamma$ , for PI-3Kinase subunits. Data are representative of two independent experimental determinations.

The potential CaM binding sites are conserved among the regulatory subunits

```

1.  p85alpha C  --HGDYTLTLRKGGNNKLIKIFHRDGGKYGFS
    p55pik CaM  KMQGDYTLTLRKGGNNKLIKIIYHRDGGK----
    p85beta Ca  KIQGEYTLTLRKGGNNKLIKVFHRDGGK----

2.  p85alpha C  LIQLRETRDQYLVNLTQKGVROKKLNEWLG--
    p85betaCaM  LMQLRKIRDQYLVNLTQKGVROKKLNEWLGIX
    p55pik CaM  -----QHLVNLNHKGVROKRLNVNLGIX

3.  p55pikCaMb -----YGEPDGAPLIRESSKKGCYACSVVA
    p85alpha C  SNRNKAENLLRGKRDGTPLVRESSEKGCYA-----
  
```

Three potential CaM binding sites in p55gamma sequence

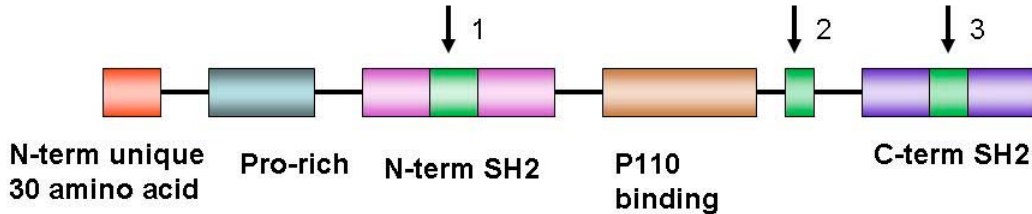


Figure 2. The putative calmodulin binding sites identified in p55gamma is conserved among regulatory subunits p85alpha, p85beta, and p55gamma.

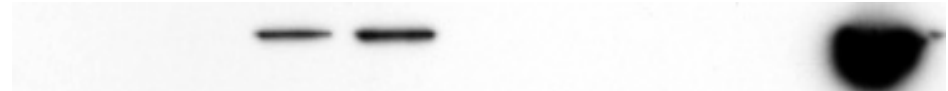

CaM sepharose	+	+	+	+		+	+
Control sepharose					+	+	
	<u>FlagSOX9</u>		<u>Flag55</u>			<u>pc55</u>	input
CaCl <sub>2</sub>	-	+	-	+	-	+	-
EGTA	+	-	+	-	+	-	+
Flag							
bound Rb							

Figure 3. Calmodulin is associated with p55gamma in a calcium-dependent way in 293T cells. The cells were transfected with Flag-tagged, non-tagged, and control plasmid (FlagSOX9). Whole cell lysates were incubated with either CaM sepharose or control sepharose in the presence or absence of 0.1mM calcium. Bound protein were detected with anti-Flag or anti-total Rb antibody.



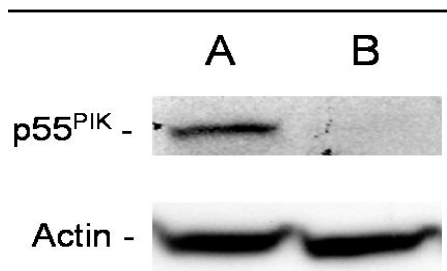
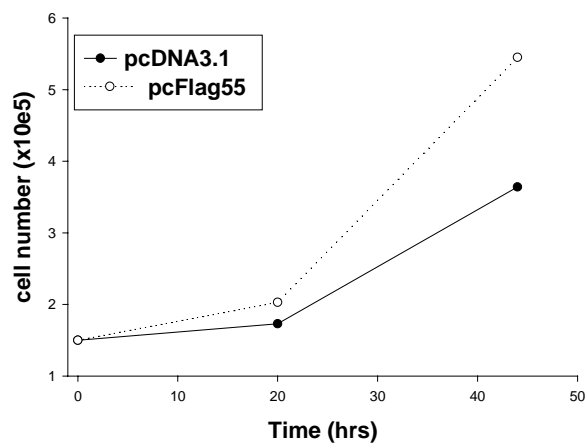
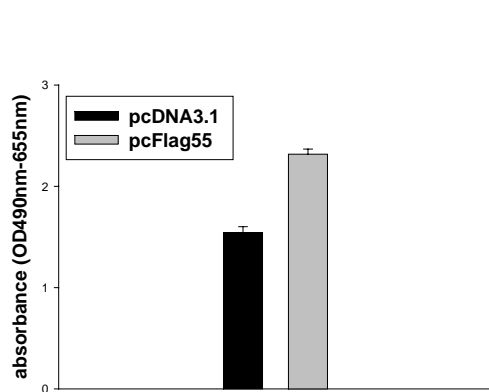
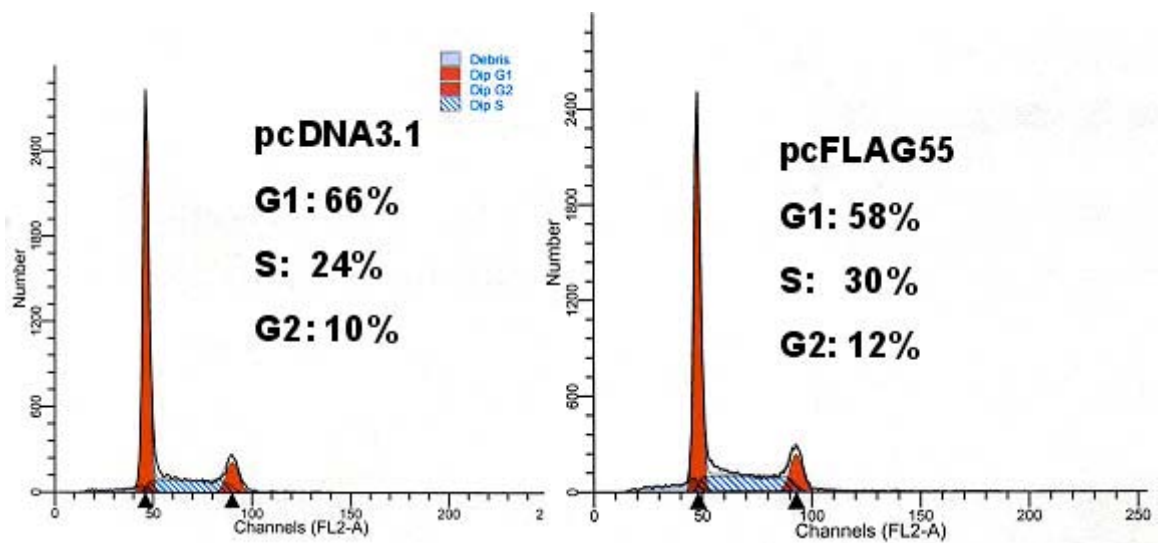
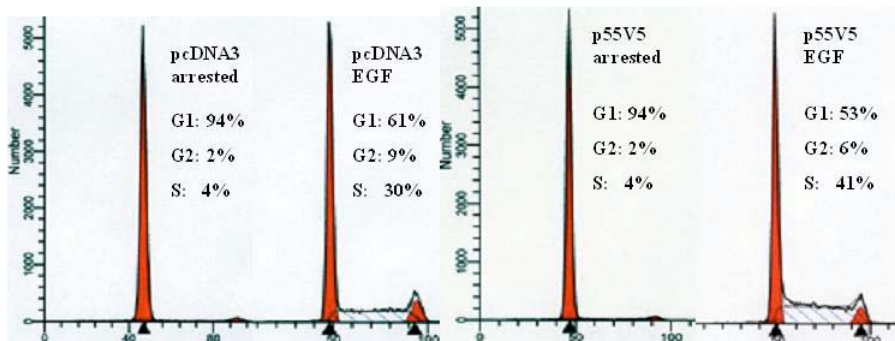


Figure 4. Knockdown of p55gamma by siRNA. AU565 cells weretransfected with non-targeting (A) or p55gamma directed siRNA (B). Lysates were prepared and blotted for p55gamma or actin.  
5a



5b

**Figure. 5. HEK293 cells stable express p55gamma demonstrate increased rate of cell cycle progression and cell proliferation. 5a. actively growing HEK293 cells overexpressing p55gamma have a greater percentage of cells in S phase compare to control parental vector transfected cells. Cell cycle histograms and percentage number of unsynchronized HEK293 cells stable expressing p55gamma or control vector grown in normal medium with selective antibiotics. 5b. p55gamma overexpressing cells show an increased proliferation rate. HEK293 Cells overexpressing p55gamma shows 50% more mitochondrial activity than control. Cell counting showed about 50% more of actively growing pcFlag55 expressing cells than control vector expressing cells.**



**Figure 6. Ectopic Expression of pc55 V5-His tagged protein increases the percentage of S phase A1N4 cells.  $10^6$  of A1N4 cells were transiently transfected with 2 micrograms of plasmid DNA and EGF starved for 48h for G1 arrest. The cells were allowed to reenter the cell cycle by replacing EGF, and were collected for FACS analysis 24 h after stimulation.**